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We have previously demonstrated that EGF activated STAT1 in some cell lines, causing cell growth arrest and apoptosis. STAT3 is often activated together with STAT1 in response to EGF. It has recently been shown that STAT3 plays an crucial role in the cell cycle progression of BAF/B03 pro-B cells. On the other hand, STAT3 activation by interleukin 6 has been demonstrated to prevent T-cells from apoptosis. More intriguingly, STAT3 but not STAT1 has been reported to be activated constitutively in several breast cancer cell lines and further enhanced in response to EGF. These recent findings led to a new hypothesis that STAT3 activated by EGF may stimulate the cell proliferation competing with activated STAT1, a negative regulator of the cell growth. Therefore, I decided to focus on STAT3 and disrupt its function in mice instead of mutating genome randomly with the frame shift mutagen. To achieve that, I employed the P1 phage-derived Cre/*loxP* site-specific recombination system since conventional STAT3 knockout mice had been shown to be embryonically lethal. Here I report on generation of mice carrying the *loxP*-flanked (floxed) (Stat3^F) or -deleted (Stat3$^-$) mutations in a germline configuration, which has been done.

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INTRODUCTION:

We have previously demonstrated that EGF activated STAT1 in some specific cell lines, causing cell growth arrest (1) and apoptosis (2) through gene induction. STAT3 is often activated together with STAT1 in response to EGF (Fig.1). It has recently been shown that STAT3 plays an crucial role in the G1 to S phase transition of BAF/B03 pro-B cells through the downregulation of p21 and p27, and the concomitant upregulation of cyclins D2, D3 and A, and cdc25A (3). On the other hand, STAT3 activation by interleukin 6 (IL-6) has been demonstrated to prevent T-cells from apoptosis (4). More intriguingly, STAT3 but not STAT1 has been reported to be activated constitutively in several breast cancer cell lines and further enhanced in response to EGF (5, 6). We have indeed observed the same phenomenon (Iwamoto, et al., data not shown). These recent findings led me to a new hypothesis that the balance of STAT1 and STAT3 activation could affect the cell growth regulation. Namely, STAT3 activated by EGF may stimulate the cell proliferation competing with activated STAT1, a negative regulator of the cell growth. Based on this hypothesis, I concluded that specific inactivation of STAT3 should be more efficient and straightforward to clarify the molecular mechanism of cell cycle regulation by STAT proteins than inducing random mutations in breast cancer cells as originally proposed in Statement of Work, Task 2. To achieve that, I employed a gene targeting technique with mice. Since conventional STAT3 knockout mice had been shown to be embryonically lethal (7), I decided to use the P1 phage-derived Cre/*loxP* site-specific recombination system (8) and disrupt the Stat3 gene in a mammary gland-specific manner. I have already generated mice carrying the *loxP*-flanked (floxed) (Stat3^F) or deleted (Stat3$^-$) mutations in a germline configuration. To achieve STAT3 inactivation specifically in mammary epithelial cells, I plan to cross Stat3^F mice with transgenic mice that express Cre under the control of the mouse mammary tumor virus (MMTV) long term repeat (LTR). The MMTV LTR is widely used to express oncogenes such as Ha-ras (9), c-myc (9), c-neu (10), v-src (11) and fgf8 (12) in mouse mammary epithelium to investigate mammary tumorigenesis. I will, then, prepare mammary epithelial primary cultures from both mutant and control mice and investigate the function of STAT proteins in cell cycle regulation and apoptosis as described in my proposal. Here I will report on generation of STAT3-floxed mice which has been done.

BODY:

Cloning of genomic Stat3 gene

Genomic clones cotaining SH2 domain of the murine Stat3 gene were isolated from a λFix II murine 129/Sv genomic library by screening the library using the StuI-ScaI fragment of the murine Stat3 cDNA as a probe. Six phage clones (clones 1, 2, 3, 4, 7 and 10) containing SH2 domain sequences of the murine Stat3 gene were identified.

Generation of the Stat3 targeting vector

The targeting vector was constructed as follows. A 3.8 Kb SmaI fragment and a 2.7 Kb NotI-SmaI fragment of clone 1 (Fig. 2) were inserted into pBluescript KS to generate pBS-1-A and pBS-1-B, respectively. DNA sequence and PCR anayses showed that pBS-1-A contained exons 20 (3' end), 21 and 22, and that pBS-1-B cotained exons 15, 16, 17, 18, 19 and 20 (5'end). SH2 domain was found to be coded by exons 19 and 20. We planed to delete SH2 domain specifically by Cre-loxP site-dependent recombination. A loxP site coupled with a HindIII restriction site was introduced into intron 20 of pBS-1-A by insertional mutagenesis using Transformer Site-Directed Mutagenesis Kit (CLONTECH, Palo Alto, CA) (Construct I). Because introns 18 and 19 are too short to introduce another loxP site without regard to interference of splicing, we produced a BglII restriction site in intron 17 of pBS-1-B by site-directed mutagenesis using the same kit as above and inserted a neomycin resistance gene cassette (neo^r) flanked by two loxP sites into the BglII site (Construct II). A SmaI fragment was excised from Construct I and inserted into the SmaI site of Construct II to get Construct III. The pBluescript SK carrying two tandem copies of HSV-thymidine kinase gene cassettes (*HSV-tk*) were digested with NotI and XhoI to recover the fragment which contains *HSV-tks* with the vector. This fragment was replaced with the vector portion of Construct III using the NotI and XhoI sites to get the final construct, pNT-ST3 (Fig. 2).

Transfection of embryonic stem cells and generation of mice

The R1 embryonic stem cell line (13) has been cultured as described. Embryonic stem cells were electroporated with 25 ug of NotI-linearized pNT-ST3 per 1 x 10^7 cells and grown under double selection as described (14). We analyzed 112 embryonic stem cell colonies for homologous recombination by Southern blotting. A genomic DNA was digested with HindIII, and two kinds of probes referred to as A and B were used. Nine clones out of 112 were positive for desirable recombination (Fig.2). Two positive clones (clones 21 and 31) were microinjected into blastocysts of B6 mice to generate mice carrying the heterozygous mutation in the germ line (Stat3$^{F-Neo/+}$).

### Removal of neo^r from the mutant allele (Stat3^{F-Neo}) in vivo

Removal of *neo^r* from the mutant allele is desirable to get a higher efficiency of Cre-dependent recombination *in vivo*. Neo^r may also exert cytotoxicity dependently on the genomic locus it is integrated in. To remove *neo^r in vivo*, we first crossed a Stat3$^{F-Neo/+}$ mouse with the transgenic mouse expressing Cre recombinase systemically including germ cells (transgenic line TTR 19-5; Iwamoto et al., unpublished) to get the Stat3$^{F-Neo/+}$ mouse carrying the transgene. The resulting mouse (Stat3$^{F-Neo/+}$; Cre$^{+/-}$) was crossed with the Stat3$^{F-Neo/+}$ mouse. The offsprings were then analyzed for Cre-mediated recombination by PCR as described below. Two offsprings out of eleven contained in the germ line the mutant allele from which only *neo^r* had been removed (Stat3^F) (Fig. 3). The rest of all nine offsprings had the mutant allele from which both *neo^r* and SH2 domain had been deleted (Stat3$^-$) (Fig. 3). Alternatively , instead of TTR 19-5, we used another Cre transgenic line, Splicer (Koni et al., unpublished), that expresses the transgene exclusively in ovaries. The same types of Cre-mediated recombination were observed with the similar frequencies.

Detection of wild-type, Stat3^{F-Neo}, Stat3^F and Stat3⁻ mutant alleles by polymerase chain reaction

Genomic DNA isolated from mouse tails was subjected to PCR for genotyping. The primer pairs a (5'-TATCACTTGCTCCACCTGCTGG-3' and 5'-GCTGGCTCATAGGCAAAAACAC-3'), b (5'-GTTGCTAAGTGTGTTTGAAGAGC-3' and 5'-ACATGTACTTACAGGGTGTGTGC-3'), and c (5'-GTTGCTAAGTGTGTTTGAAGAGC-3' and 5'-GCTGGCTCATAGGCAAAAACAC-3') were used to detect Stat^F, Stat^F and wild-type, and Stat3⁻ alleles, respectively (Fig.3). The allele carrying the *neo^r* cassette can be detected by the primer pair d (5'-CCTCAGAAGAACTCGTCAAGAAGG-3' and 5'-GCCAATATGGGATCGGGCCATTGA-3'). The Stat3^{F-Neo} allele can be identified as the one which are positive for both Stat^F and *neo^r* (Fig.3).
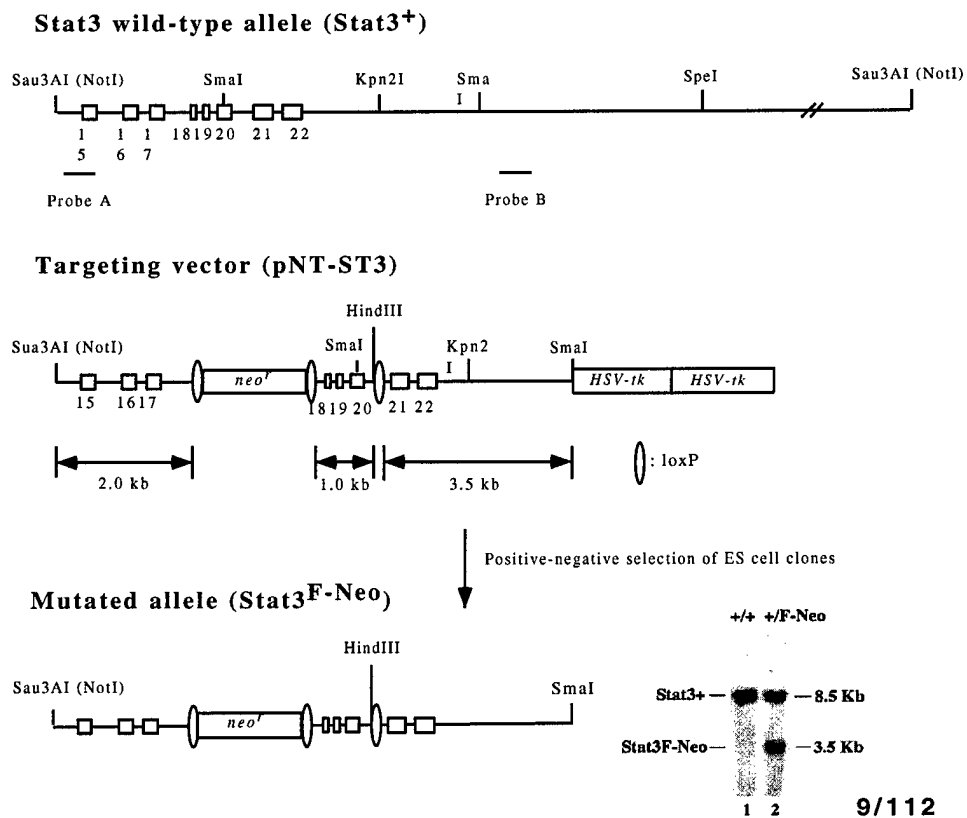
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A431

| EGF | – – + |
| IFN-γ | – + – |

SIF-A
SIF-B
SIF-C

Probe: M67-SIE —

1 2 3
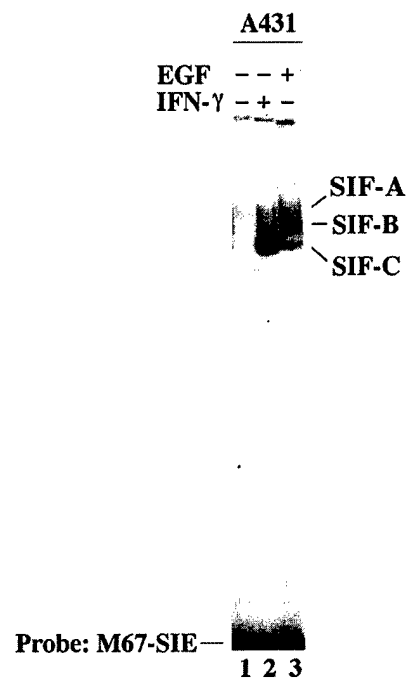
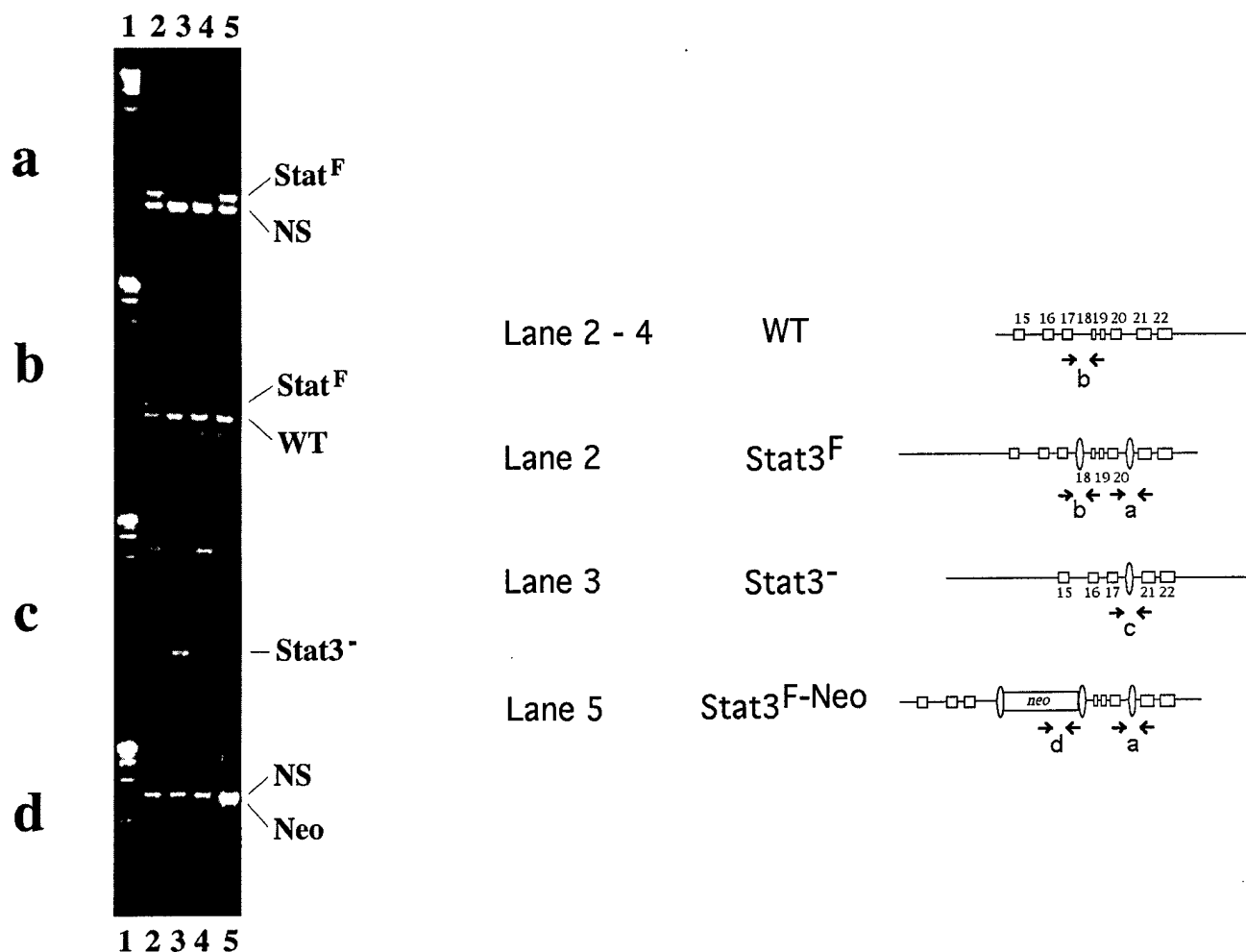**Figure 1. Activation of STAT proteins by EGF in A431 cells.**

A431 cells were deprived of serum for 17 h and stimulated with 100 ng/ml EGF or
1 ng/ml interferon-γ for 15 min. One microliter of the whole cell extract was
subjected to EMSA using M-67 SIE as a probe. Three types of the STAT protein-SIE
complexes (SIF-A, B and C) were observed. SIF: STAT3 homodimer;
SIF-B: STAT1/STAT3 heterodimer; SIF-C: STAT1 homodimer.

Stat3 wild-type allele (Stat3$^+$)

Sau3AI (NotI) SmaI Kpn2I Sma I SpeI Sau3AI (NotI)

1 5 1 6 1 7 18 19 20 21 22

Probe A

Probe B

Targeting vector (pNT-ST3)

HindIII

Sua3AI (NotI) SmaI Kpn2 I SmaI

neo^r

15 16 17 18 19 20 21 22 HSV-tk HSV-tk

2.0 kb

1.0 kb

3.5 kb

◯ : loxP

Positive-negative selection of ES cell clones

Mutated allele (Stat3^{F-Neo})

HindIII

+/+ +/F-Neo

Sau3AI (NotI) SmaI

neo^r

Stat3+ — —8.5 Kb

Stat3F-Neo— —3.5 Kb

1 2   9/112

Figure 2. Targeting of loxP sites into the Stat3 locus of ES cells
(R1)

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Figure3 . PCR genotype analysis of the Stat3 gene.

Left panel: Agarose gel electrophoresis of PCR products.
The genotypes for the samples are Stat3$^{F/+}$ (lane 2), Stat3$^{-/+}$ (lane 3), Stat3$^{+/+}$ (lane 4) and Stat3$^{F-Neo/+}$ (lane 5). The letters a, b, c and d beside the panel demonstrate pairs of PCR primers used for each reaction. These primers were designed against the regions represented in the right panel.

Right panel: Schematic representation of wild-type and mutant Stat3 alleles.
The PCR product from the wild-type Stat3 allele (WT) is observed in lanes 2-5.
The products from Stat3^{F} , Stat3^{-} and Stat3^{F-Neo} alleles are observed in lanes 2, 3 and 5, respectvely.

APPENDICES:

1) Key research accomplishments:
    a. Cloning of genomic Stat3 gene
    b. Making the targeting vector, pNT-ST3
    c. Selection of ES cell clones which are positive for homologous recombination
    d. Generation of the transgenic line (TTR 19-5) expressing Cre recombinase systemically
    e. Generation of Stat3-floxed (Stat3F) and -deleted (Stat3-) mice

2) Reportable outcomes:

    i) Developed reagents:
        a. ES cell lines were developed that carry a floxed-mutation in the Stat3 genomic locus
        b. The transgenic line (TTR 19-5) expressing Cre recombinase systemically
        c. Stat3-floxed (Stat3F) and -deleted (Stat3-) mice

    ii) Funding applied for based on work supported by this award:
        a. Career Development Awards of U.S. Army 1999 Breast Cancer Research Program

    iii) Employment received based on experiences/training supported by this award:
        a. I was offerred a position of Research Assistant Professor at the Mount Sinai School of Medicine.